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Pattern of Variation and Systematics of *Nymphaea odorata*: I. Evidence from Morphology and Inter-Simple Sequence Repeats (ISSRs)

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Abstract. *Nymphaea odorata*, Nymphaeaceae, is the most widely distributed water-lily in North America. Disagreement exists on whether this morphologically variable species should be split into two species, *N. odorata* and *N. tuberosa*, or treated as one species with two subspecies. Morphological characters and markers from the inter-simple sequence repeats (ISSRs) were examined to assess taxonomic status and elucidate patterns of genetic variation among populations. This study provides evidence against treatment of *N. tuberosa* at species rank. The principal component analysis of 26 vegetative characters underscores immense variability, but does partially segregate populations of subsp. *odorata* and subsp. *tuberosa*. Based on analysis of variance, a new set of morphological characters is proposed to distinguish the two subspecies: mean leaf blade length-to-width ratio, petiole striping, and lobe apex shape. Results from ISSRs show high polymorphism within and among populations. Genetic variation was found largely within geographical regions (89%) rather than among regions. Principal coordinate (PCOA) analyses and minimum spanning tree (MST) analyses based on ISSRs clearly distinguished *Nymphaea mexicana* and *N. odorata*. Within *N. odorata*, samples of subsp. *odorata* appear to be a distinct entity, whereas samples largely but not completely separated from samples of subsp. *tuberosa*. PCOA and MST showed linkage between most samples of subsp. *odorata* whereas this was less evident in UPGMA.

Nymphaea odorata Aiton is the most common water-lily in North America, ranging from Florida north to Nova Scotia and Newfoundland, and west to Manitoba, Nebraska, Texas, and central Mexico. A few populations exist elsewhere in Central America and the Greater Antilles (Wiersema and Hellquist 1997). This important peat-forming species (Casagrande et al. 1980) is unique in the genus in its production of extensive horizontal rhizomes, often covering large areas. Self pollination in this water-lily species has not been observed (Schneider and Chaney 1981), and studies that quantify the extent of vegetative vs. sexual reproduction in natural populations are lacking. *Nymphaea odorata* belongs to the temperate subg. *Nymphaea* (Wiersema 1996). The subgenus appeared monophyletic in analyses of phylogenetic relationships in *Nymphaea* using *trnT-trnF* and ITS sequence data (Borsch 2000). Within this clade, *N. mexicana* Zucc. has either been inferred as the first branch or, in some analyses of ITS with denser sampling, a clade consisting of *N. mexicana* and *N. odorata* appeared sister to the rest of subgenus *Nymphaea*. Statistical support for either of the two hypotheses was weak. Therefore, it is not yet clear if *N. odorata* and *N. mexicana* share an immediate common ancestor, or if *N. odorata* shares a common ancestor with the Eurasian and boreal species of subg. *Nymphaea*. Morphological characters, however, support the first branching position of *N. mexicana* (Borsch et al. unpubl. data). Moreover, natural hybrids between *N. mexicana* and *N. odorata* have been reported (Ward 1977; Wiersema 1983), which can usually be identified by phenotypes intermediate between the parents. Thus several populations of *N. mexicana* were also included in this ISSR analysis, to detect possible introgression into *N. odorata*.

The taxonomic status of the *Nymphaea odorata-tuberosa* complex has been disputed. The two have been recognized at the species level (Conard 1905; Fernald 1950; Correll and Correll 1975; Godfrey and Wooten 1981). However, Wiersema and Hellquist (1994) indicated that apparent plasticity over the wide geographic distribution and intergradations in morphology along areas of overlap blurs the distinctness between the two. Consequently, the two species were lumped as two subspecies under *N. odorata*, subsp. *odorata* and subsp. *tuberosa* (Paine) Wiersema & Hellquist, based on morphological characters and geographical distribution (Wiersema and Hellquist 1997). Wiersema and Hellquist (1997) distinguished subspecies *tuberosa* from subsp. *odorata* on the basis of petiolar stripes, green leaf blade undersurface, and 2.8–4.5 mm long seeds. This subspecies extends in distribution from the central United States north into Canada (Fig. 1). In contrast, subsp. *odorata* generally lacks petiole striping, has a reddish-purple leaf blade undersurface, and bears 1.5–2.5 mm long seeds. It has a wide geographic distribution in North America, encompassing major portions of the United States and extending into Canada

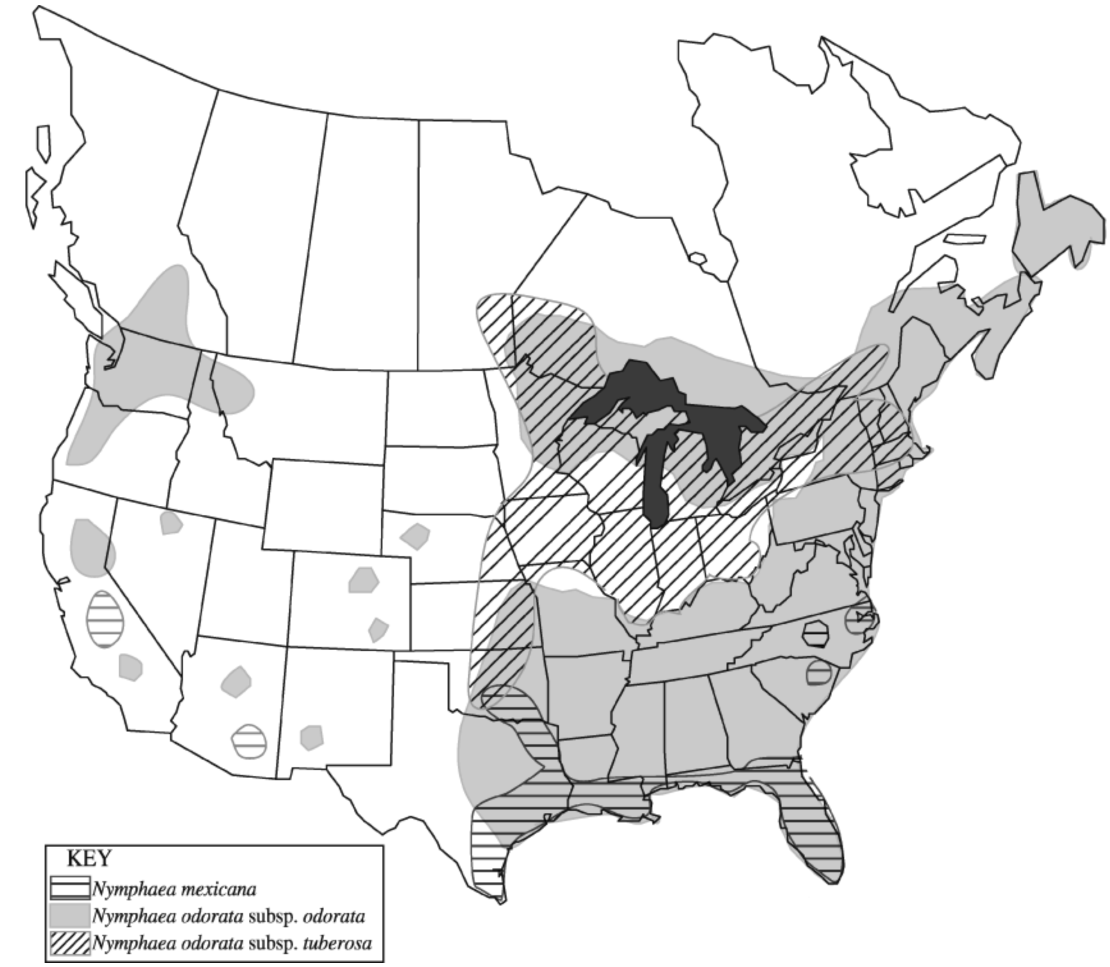

FIG. 1. The geographic distribution of *N. mexicana* and *N. odorata* in North America. *Nymphaea odorata* subsp. *odorata* has the widest distribution, overlapping with both *N. mexicana* and subsp. *tuberosa* (Wiersema and Hellquist 1997).

(Fig. 1). In addition to these subspecies, several varieties have been recognized (Conard 1905; Ward 1977; Godfrey and Wooten 1981; Harvill et al. 1992) that are now subsumed under subsp. *odorata* (Wiersema and Hellquist 1997).

Some of the morphological characters distinguishing the subspecies in *Flora of North America* (*FNA*), such as petiolar stripes and color of the abaxial leaf blade surface, were observed to vary considerably throughout the range of each subspecies (Wiersema and Hellquist 1997). Such difficulties in segregation have been attributed to gene flow between the subspecies, and suspected fertile hybrids have been found in Canada, Minnesota, Michigan, New York, Vermont, and Wisconsin, where subspecies distributions overlap (Wiersema and Hellquist 1994; Wiersema and Hellquist 1997). These putative hybrids combine characters from both parents and possess intermediate character states, which complicate the morphologically based distinction between the two subspecies.

The extent and pattern of variation in morphologies and the degree of overlap in this species complex have not been quantitatively studied and non-morphological information has not been applied to this taxonomic problem. In this study we use information from morphology and the inter-simple sequence repeat markers (ISSR) to test the above taxonomic hypotheses. Patterns of genetic variation have been successfully assessed in population studies using ISSRs markers (e.g., Raina et al. 2001). ISSR markers are based on primers that anneal to microsatellite motifs of one to three nucleotides, such as $(CA)_x$. ISSRs are considered to be reproducible markers that provide sufficient characters for assessment of variability, and are at the same time cost effective (Wolfe and Liston 1998). Compared to other fingerprinting techniques such as RAPDs, ISSRs

TABLE 1. Samples used along with their geographic origin and collector information. Samples in bold were used in morphological analyses; those containing a sample number were used in molecular analyses. Voucher specimens were deposited at BONN, FR, FTG, MEXU and VPI (herbarium abbreviations follow Holmgren et al. 1990). Data are presented in the following sequence: *Nymphaea* species, Sample #, Geographic Origin, Voucher Information.

N. mexicana, 69N, Florida, *Borsch & Summers 3227* (BONN); KN8, Texas, *Woods & Borsch 0701* (VPI, BONN); KN9, Louisiana, *Woods & Borsch 1101* (VPI, BONN); KN21, Mexico, *Novelo, R.A. et al 1343* (MEXU)

N. cf. mexicana, KN20, Florida, *Borsch & Summers 3213* (FR); KN22, Florida, *Borsh & Summers 3214* (FR)

N. odorata subsp. *odorata*, Maryland, ***Borsch, Hilu & Wiersema 2361*** (VPI, BONN); 11N, Florida, *Borsch & Wilde 3128* (FR); 42N, Florida, *Borsch & Wilde 3099* (FR); 33N, Florida, *Borsch & Wilde 3101* (FR); 34N, Florida, *Borsch & Wilde 3125* (FR); 35N, Florida, *Borsch & Wilde 3127* (FR); 37N, Georgia, *Borsch & Wilde 3131* (FR); 38N, Georgia, *Borsch & Wilde 3133* (FR); 39N, Georgia, *Borsch & Wilde 3134* (FR); 40N, Georgia, *Borsch & Wilde 3135* (FR); KN7, Michigan, ***Borsch, Wiersema & Helquist 3398*** (VPI, BONN); KN10, Texas, ***Woods & Borsch 0801*** (VPI, BONN); KN11, Louisiana, ***Woods & Borsch 0901*** (VPI, BONN); KN12, Louisiana, ***Woods & Borsch 1001*** (VPI, BONN); KN18, South Carolina, ***Woods & Wiersema 0601*** (VPI); KN19, Virginia, ***Woods 1201*** (VPI); KN23, Vermont, ***Borsch, Wiersema & Hellquist 3331*** (VPI, BONN); KN24, North Carolina, ***Woods 1401*** (VPI); KN26, Tennessee, ***Woods & Neves 1701*** (VPI); KN27, Vermont, ***Borsch, Wiersema & Hellquist 3322*** (VPI, BONN); KN29, Vermont, *Borsch*, ***Wiersema & Hellquist 3330*** (VPI, BONN); KN30, Florida, *Borsch & Summers 3215* (FR); KN32, Vermont, ***Borsch, Wiersema & Hellquist 3323*** (VPI, BONN); KN33, Vermont, *Borsch, Wiersema & Hellquist 3324* (VPI, BONN); KN16, Delaware, ***Woods & Wiersema 0401*** (VPI); KN25, Tennessee, ***Woods & Neves 1501*** (VPI); KN37, Virginia, ***Woods 1301*** (VPI)

N. odorata subsp. *tuberosa*, KN28, Vermont, ***Borsch, Wiersema & Hellquist 3329*** (VPI, BONN); Michigan, ***Borsch & Wiersema 3400*** (VPI, BONN); KN5, Wisconsin, *Borsch, Wiersema & Hellquist 3396* (VPI, BONN); Vermont, ***Borsch, Wiersema & Hellquist 3326*** (VPI, BONN); 1N, New York, *Borsch 3156* (BONN); KN6, Manitoba, Canada, ***Borsch, Wiersema & Hellquist 3389*** (BONN); Manitoba, Canada, ***Borsch, Wiersema & Hellquist 3392*** (VPI, BONN); KN14, Michigan, ***Woods & Wiersema 0201*** (VPI); KN15, Ohio, ***Woods & Wiersema 0301*** (VPI); KN13, Pennsylvania, ***Woods & Wiersema 0101*** (VPI); KN31, Vermont, ***Borsch, Wiersema & Hellquist 3325*** (VPI, BONN); KN17, Ohio, ***Wiersema 2384*** (VPI)

N. tuberosa Paine S.M., Lake Ontario, New York, *Lectotype* ***Cat. Pl. Oneida Co., 132. 1865***

N. odorata s. l., KN1, Michigan, *Borsch & Wiersema 3399* (VPI); KN3, Michigan, *Borsch & Wiersema 3401* (VPI); KN4, Michigan, *Borsch & Wiersema 3402* (VPI)

have been shown to be more reliable (Qian et al. 2001), which may be due to longer primers that are annealing at higher temperatures. Each ISSR fragment size is treated as a locus and the band represents a diallelic marker (Wolfe and Randle 2001).

The specific objectives of this study were (1) to quantitatively evaluate the extent and patterns of morphological variability of the two subspecies and to determine the degree of their differentiation using univariate and multivariate statistical approaches, (2) to statistically test whether the character states of the individual samples support the subspecific grouping as defined in FNA, and (3) to assess the genetic diversity and relationships among populations of *N. odorata* using ISSR markers. This study is complemented by a sequence-based approach using ITS and *trnL-trnF* regions (Woods et al. 2005).

MATERIALS AND METHODS

Sampling Strategy. Several field trips were conducted from 1997 to 2001 to gather samples of the *N. odorata* complex and *N. mexicana* from across their North American ranges for both the morphological study and the ISSR analysis. Several samples per population were collected in a few cases to assess intrapopulation variability. In addition, several herbarium specimens were used.

Morphological Analyses. Thirty samples of *N. odorata* (Table 1) were examined for 26 morphological characters (Table 2). Measurements were taken from herbarium samples or dried specimens collected from field trips. Samples were identified (Table 1) on the basis of morphological characters listed in Wiersema and Hellquist (1997). Samples that could not be keyed out to either subspecies because of possession of intermediate phenotypes were treated as *N. odorata* without subspecies affiliation. Quantitative and qualitative morphological characters (Table 2) include those used in existing taxonomic treatments of *N. odorata* (Conard 1905; Radford et al. 1968; Godfrey and Wooten 1981; Wiersema and Hellquist 1997; Borman et al. 1999). Qualitative characters were scored as binary states (present/absent) or unordered multistate

TABLE 2. Qualitative and quantitative morphological characters measured for each sample. Scoring of qualitative characters is following the character listed. Blade length was measured from the top of the leaf blade to the sinus.

Qualitative Characters. 1. Petiole stripes: 1 = present; 0 = absent. **2. Petiole hairs**: 1 = present; 0 = absent. **3. Sinus overlap**: 1 = present; 0 = absent. **4. Leaf apex**: 1 = retuse; 0 = rounded. **5. Lobe apex**: 1 = ovate; 0 = rounded. **6. Color of the leaf blade undersurface**: 2 = red; 1 = reddish green; 0 = green. **7. Tubers**: 1 = present; 0 = absent. **8. Sepal apex**: 1 = ovate; 0 = rounded. **9. Sepal veins**: 1 = apparent; 0 = not apparent. **10. Longest petal widest point**: 2 = top 1/3; 1 = middle; 0 = bottom 1/3. **11. Longest petal apex**: 1 = pointed; 0 = rounded. **12. Shortest petal apex widest point**: 2 = top 1/3; 1 = middle; 0 = bottom 1/3

Quantitative Characters. 1. Petiole diameter. 2. Blade width. 3. Blade length. 4. Blade length/blade width. 5. Blade sinus length/blade length. 6. Blade sinus length. 7. Number of leaf veins. 8. Rhizome diameter. 9. Peduncle diameter. 10. Sepal length. 11. Sepal width. 12. Number of petals. 13. Longest petal length. 14. Longest petal width.

TABLE 3. Comparison of ISSR primers used for interpopulation analysis on 43 samples from *N. odorata* and *N. mexicana.* The percentage of polymorphic bands were calculated as the number of polymorphic loci per group (subsp. *tuberosa,* subsp. *odorata, N. mexicana*) divided by the total number of loci from that primer. * = primer that displayed intrapopulation variation.

				% bands polymorphic:		
Primer	Sequence	# loci	# Genotypes	Subsp. *tuberosa* (n = 13)	Subsp. *odorata* (n = 24)	*N. mexicana* and *N. cf. mexicana* (n = 6)
843	$(CT)_8$-RA	16	35	88	88	6
899	$(CA)_6$-RG	14	36	79	100	71
901	$(GT)_6$-YR	17	30	70	82	88
CA8	$(CA)_8$-RG	13	34	77	92	38
814*	$(CT)_8$-TG	12	41	83	92	75

characters (Table 2), whereas quantitative characters were either measured or counted and scored as continuous characters. Blade length was measured from the top of the leaf blade to the sinus.

Multivariate methods were used to determine if a grouping of samples into two entities could be constructed without *a priori* assumption of subspecies classification (Boonkerd et al. 2002). Analyses were based on four different data sets extracted from the raw data set: (1) all morphologic characters, (2) qualitative characters alone, (3) quantitative characters alone, and (4) characters that were significant at $p < 0.10$ level in an analysis of variance (ANOVA). Sequential, agglomerative, hierarchal, and nested (SAHN) clustering, principal coordinate (PCOA) and minimum spanning tree (MST) analyses were performed on the four matrices (all 30 samples included). SAHN clustering was performed using the unweighted pair-group method with arithmetic averages (UPGMA). Matrices of similarities were generated from the raw data of matrices 1, 3 and 4, whereas for matrix 2, comprised entirely of qualitative characters, the simple matching coefficient was used. All these analyses were performed in NTSYS-pc computer program version 2.102 (Rohlf 1998).

Discriminate analysis, multiple analysis of variance (MANOVA), and principle component analysis (PCA) were performed on the 25 samples of *N. odorata* with complete information using SAS version 8.0 (SAS Institute 1999–2001). PCA was used to evaluate patterns of variation in populations of *N. odorata* and the characters that contribute most to these patterns. A discriminate analysis assesses the degree of discrimination among clusters (or taxa) and the characters that are most effective in separating them. The MANOVA provides further assessment of patterns of variation in characters. In these analyses, samples with missing values (KN26, KN10, KN18, KN14, *N. tuberosa* lectotype) were excluded, as were any variables with few entries (qualitative characters 7–12 and quantitative 8–14; Table 2) . All remaining quantitative variables were log (base 10) transformed because they were not normally distributed. The PCA was based on a correlation matrix, whereas discriminant analysis and MANOVA were performed on only quantitative characters 1–7 because these analyses are designed for quantitative data and do not accept missing values (Table 2). The MST was superimposed on the PCOA to emphasize relationships between nearest neighbors and reveal possible distortion in the PCOA.

Evaluation of the degree to which each character is contributing to the discrimination between subspecies was conducted using univariate and multivariate statistical approaches. Univariate analysis was performed on each character to determine if a statistically significant difference existed between subspecies as classified on the basis of characters defined in Wiersema and Hellquist (1997). An ANOVA was generated for each morphological character using a student's t-test for quantitative characters and a contingency table for qualitative characters. All univariate analyses were performed in JMP IN, version 4 (SAS Institute, Inc.).

In univariate analysis, MANOVA, and discriminate analysis, *a priori* decision on groupings is a prerequisite. Samples identified as *N. odorata* without subspecies affiliation were included with subsp. *tuberosa* (three samples from the same population) based on results from a molecular analysis of the internal transcribed (ITS) region (Woods et al. 2005).

ISSR Analyses. Ten ISSR primers (Promega, Madison, WI) were initially scanned on ten samples from a Tennessee population (data not shown). This pilot study was conducted to determine the degree of intrapopulation variation and evaluate the effectiveness of primers in resolving interpopulation differences in *N. odorata*. Of these ten primers, four primers that did not reveal variability within the Tennessee population were chosen to assess interpopulation variation (Table 3). A fifth primer (Primer 814), that resolved one of the highest level of intrapopulation variation, was used to determine if primers variable within a population could be applied to the interpopulation study.

Thirty-seven samples representing both subspecies of *N. odorata*, two samples tentatively identified as *N. cf. mexicana,* and four samples of *N. mexicana* were examined (Table 1). DNA was extracted from silica gel-dried or frozen leaf tissue by a modified CTAB method (Borsch et al. 2003). ISSRs were amplified with a single primer using the polymerase chain reaction (PCR) and a PTC-100 thermocycler (MJ Research, Inc., South San Francisco, California). Reactions were conducted in a 25 μL mixture consisting of 0.4 μ*M* primer, 1X *Taq* polymerase buffer, 0.2 *M* dNTPs, 0.2 u *Taq* DNA polymerase (Promega, Madison, Wisconsin), 1.5 mM $MgCl_2$, and 1 μL of DNA. Thermocycler protocol followed Wolfe and Randle (2001). Replicate experiments were performed to check the reproducibility of ISSR banding patterns. Additionally, all experiments included negative controls by replacing DNA with sterile water.

The PCR products were analyzed on 1.5% agarose gels in 1X TAE buffer following the conditions of Wolfe and Randle (2001). Gels were stained with ethidium bromide and visualized using Alpha Digital Image System (Alpha Innotech Corp., San Leandro, California). Digital images of each gel were printed in 20 × 27.5 cm format and fragment sizes were calculated by comparing the samples to a 1 kb ladder standard (Promega, Madison, Wisconsin) included in the gels. ISSR data were scored as band present (1) or absent (0) across samples for each primer. Only reproducible bands were scored for data analysis.

For each primer, the number of bands and the number of genotypes (genotype = unique banding pattern) were determined. Additionally, the percentage of polymorphic bands was calculated for three taxonomic groups: (1) *N. odorata* subsp. *tuberosa*, (2) *N. odorata* subsp. *odorata* and (3) *N. mexicana* and *N. cf. mexicana.* The percentage of polymorphic bands was calculated as the number of polymorphic loci per taxonomic group (subsp. *tuberosa*, subsp. *odorata*, *and N. mexicana*) divided by the total number of loci in the primer (Li and Ge 2001).

The matrix constructed from the presence/absence of ISSR bands was utilized to generate a similarity matrix with the DICE coefficient (Pearson et al. 2002), and the latter analyzed with the UPGMA method to evaluate patterns of population clustering. To examine the patterns of variation among samples, a PCOA was performed based on a DICE (Pearson et al. 2002) similarity matrix. To detect potential local distortion in the PCOA, an MST was computed and superimposed on the similarity matrix. All analyses were performed in NTSYS-pc computer program version 2.102 (Rohlf 1998).

An analysis of molecular variance (AMOVA) was computed to determine the genetic structure within and among groups. Initially, the presence/absence matrix was input into AMOVA prep

TABLE 4. ANOVA analyses of the morphological variables that differ among the subspecies (subsp. *odorata* and subsp. *tuberosa*). R^2 = correlation coefficient; and P = significance value obtained using a student's t-test for quantitative variables and a contingency table for qualitative variables.

Character	R^2	P
Petiole stripes	0.54	0.001
Blade length/blade width	0.22	0.008
Shortest petal length/shortest petal width	0.13	0.01
Petiole hairs	0.16	0.03
Blade sinus length	0.16	0.03
Lobe apex	0.2	0.05
Blade sinus length/blade length	0.12	0.06
Number of leaf veins	0.11	0.07

computer program version 1.55 (Miller 1998) to create a distance matrix. This matrix was input in the AMOVA computer program version 1.55 (Excoffier 1995) to estimate the variation among individuals within regions, among regions within species, and among species (Excoffier et al. 1992). Samples from an individual state in the United States (Table 1) constituted a region and species were defined as the three taxonomic groups (subsp. *odorata*, subsp. *tuberosa*, *N. mexicana*).

RESULTS

Morphological Study. Based on ANOVA, eight of the 26 vegetative morphological characters are moderately significant ($P < 0.10$) between the subspecies (Table 4). Of these, seven were derived from leaf blade characteristics, whereas the eighth was a character of petal morphology. MANOVA analysis based on quantitative characters 1–5 (Table 2) was not statistically significant (Wilks' Lambda = 0.64; F = 1.45; df = 7; P = 0.245).

SAHN clustering, PCOA, and MST based on each of the four morphological data matrices (all characters, quantitative characters, qualitative characters, and statistically significant characters) did not result in a significant grouping by subspecies or geography (data not shown). However, PCA revealed partial segregation of the two subspecies (Fig. 2). The first two PCA coordinates separate samples of subsp. *odorata* from samples of subsp. *tuberosa*. The first three derived components account for 66% of the total morphological variance (Table 5). Most of the variation in component 1 is contributed by quantitative leaf blade characteristics (blade width, blade length, blade sinus length, petiole diameter), whereas most of the variation in component 2 is contributed by qualitative leaf blade characteristics (petiole stripes, petiole hairs, and sinus overlap). Component 3 has low loadings for most variables, except for lobe apex. Discriminant analysis also revealed appreciable separation between subspecies. The discriminant analysis based on quantitative characters

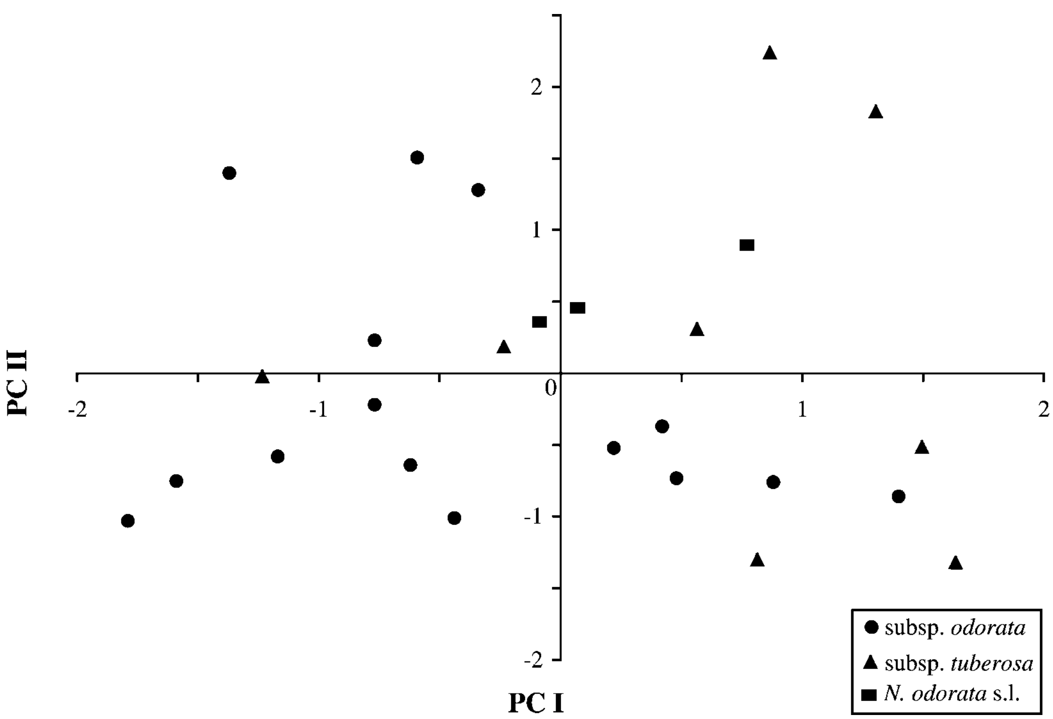

FIG. 2. Plot of scores by principal component axes I and II from 26 samples of *N. odorata* based on eleven morphological characters. Samples from subsp. *odorata* occur mostly on the negative side of both axes, whereas samples from subsp. *tuberosa* occur mostly on the positive side of both axes. Samples labeled *N. odorata* s.l. were those which could not be identified morphologically to a subspecies.

TABLE 5. Results of principal components analysis based on eleven morphological characters of 26 *N. odorata* samples. Characters arranged in descending order according to loadings on first component.

	1	2	3
Eigenvalues	3.91	2.01	1.34
Component loadings			
Blade width	0.970	−0.100	0.037
Blade length	0.934	−0.126	0.080
Blade sinus length	0.933	−0.105	0.210
Petiole diameter	0.858	0.079	−0.281
No. leaf veins	0.581	−0.145	0.106
Leaf apex	0.295	0.086	−0.606
Petiole hairs	0.187	0.753	−0.301
Lobe apex	0.127	0.243	0.814
Sinus overlap	0.010	0.670	−0.082
Color leaf blade undersurface	0.059	0.530	0.003
Petiole stripes	0.046	0.768	0.270
Percent of total variance explained	35.57	18.36	12.21

1–5 (Table 2) identified 71% of subsp. *odorata* and 67% of subsp. *tuberosa* correctly with an overall average error rate of 31%.

ISSR Study. The PCR fragments ranged from 300–2,000 bp. Seventy-two loci were scored for the five primers, which result in 30–41 genotypes for each primer (Table 3). A wide range in the percentage of polymorphic bands within and between taxa is observed (Table 3). Primer 814 produces the largest number of genotypes, 41, even though it resolved the smallest number of loci. This primer reduced resolution in the analyses when combined with other primers due to high interpopulation polymorphism and, thus, was excluded from all further analyses.

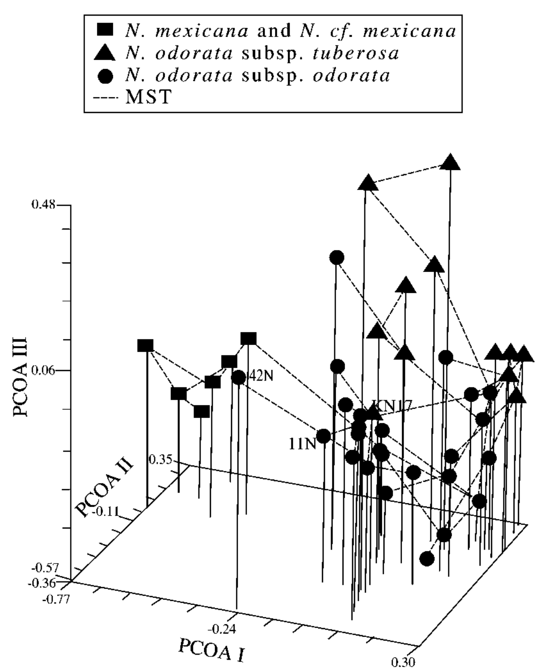

FIG. 3. Principal coordinate analyses with minimum spanning tree superimposed, based on ISSR markers excluding primer 814. *Nymphaea mexicana* and *N. odorata* are clearly distinguished; separation between the subspecies of *N. odorata* is less distinct but still evident. Samples KN17 and 42N have anomalous placement (see text). The proportion of total variance comprising each axis was 17.8% for axis I, 9.2% for axis II, and 7.2% for axis III.

The PCOA and MST resolved *N. mexicana* and *N. odorata* samples in two discrete groups (Fig. 3). Within the *N. odorata* group, further segregation in the two subspecies is also evident. MST shows primarily within-group closest neighbor linkage except for some samples of subsp. *odorata*. Sample 42N of subsp. *odorata* (Florida) is isolated from other samples on the PCOA, particularly on the second axis. However, MST linked this sample with another sample of subsp. *odorata* (11N). Sample KN17 of subsp. *tuberosa* (Ohio) is nested in the center of subsp. *odorata* samples.

Results from the UPGMA analysis of the ISSR markers (Fig. 4) are congruent for the most part with those obtained from PCOA. All samples of *N. mexicana* emerged in one cluster that was linked with *N. odorata* sample 42N at a low similarity coefficient. The analysis also depicted three of the samples from Michigan in a cluster, distant from remaining *N. odorata*. Although discrete segregation between subsp. *odorata* and subsp. *tuberosa* is not apparent, the clusters resolved (summarized as triangles in Fig. 4) by the UPGMA each encompasses members of one subspecies, with only a few exceptions. Some geographic pattern is apparent, such as the genetic affinities among five samples of subsp. *odorata* from Florida and Georgia and samples from Virginia and Tennessee.

The AMOVA shows higher variation within a geographic region than between different taxonomic groups (Table 6). Of the total genetic diversity, 14% is attributed to differences among regions within a taxonomic group and 89% to differences within a region,

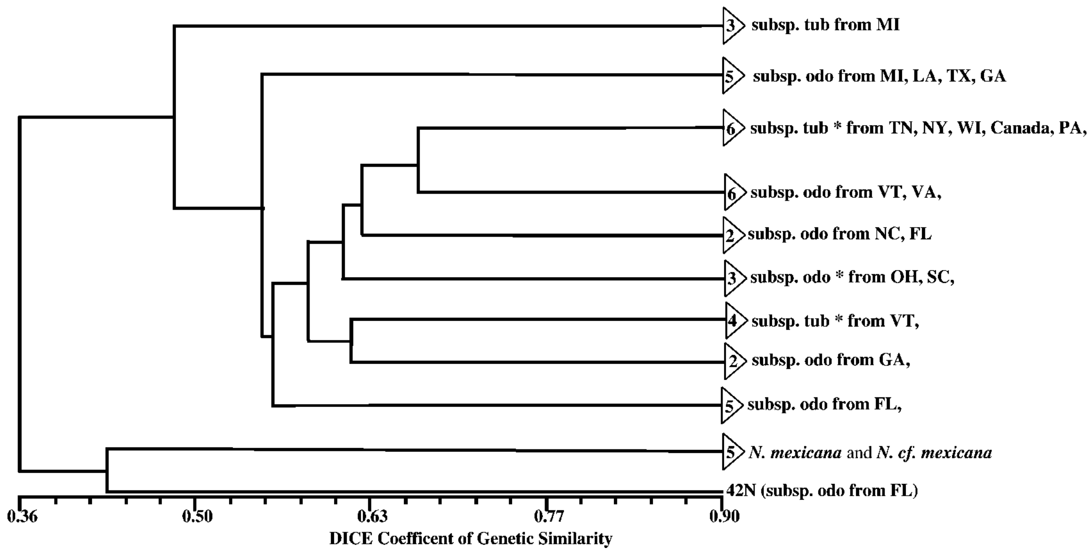

FIG. 4. Phenogram illustrating genetic relationships between *N. mexicana* and *N. odorata* generated by the UPGMA calculated from ISSR markers excluding primer 814. *Nymphaea mexicana*, the putative hybrids and *N. odorata* appear as distinct groups. Within *N. odorata*, each subspecies is represented by three distinct groups, reflecting geographic partitioning. Numbers in triangles denote the number of samples in each triangle. Geographic locations are indicated by state abbreviations. * = group includes one sample from the opposing subspecies.

with −3% attributed to differences among taxonomic groups.

DISCUSSION

Morphology. The ANOVA, MANOVA, and discriminant analyses were based on current concepts of subspecies classification, and were performed on reduced sets of the original data that excluded missing data and included only quantitative characters. In contrast, SAHN, PCOA, MST, and PCA analyses were based on a combination of quantitative and qualitative characteristics and did not require *a priori* assumptions of taxonomic affinities of samples. The univariate analysis of the 26 morphological characters shows that among the eight characters that are statistically significant between the subspecies, only petiole stripes was used to discriminate between the subspecies by Wiersema and Hellquist (1997). Seed size, one of the characters used by Wiersema and Hellquist, could not be measured here because it was available for only a few samples. Color of the leaf blade undersurface, a trait often used to distinguish the subspecies, was not statistically significant. This trait varied considerably, and in 12 samples from both subspecies, the leaves have a mix of reddish-purple (subsp. *odorata*) and green (subsp. *tuberosa*) color. The majority of the statistically significant morphological characters were leaf blade characteristics (Table 4) that have not been previously emphasized in the taxonomy of *N. odorata*. Earlier treatments of *N. odorata* (Conard 1905; Radford et al. 1968; Godfrey and Wooten 1981; Harvill et al. 1992; Wiersema and Hellquist 1997; Borman et al. 1999) have not used leaf blade shape, length of sinus and number of leaf veins as taxonomic characters.

We used the means of the statistically significant characters of the leaf blade (Table 4) to generate a typical leaf blade for each subspecies (Fig. 5). Based on leaf characteristics, each subspecies can be distinguished on the basis of leaf blade length/width ratio, depth of blade sinus, number of leaf veins, and shape of lobe apex. The leaves of subsp. *tuberosa* are more orbicular in shape than those of subsp. *odorata* in the

TABLE 6. Analysis of molecular variance (AMOVA) based on ISSR markers excluding primer 814. The total data set contains three taxonomic groups (subsp. *odorata*, subsp. *tuberosa* and *N. mexicana*) and 19 regions (which represent individual states). Statistics include sums of squared deviations (SSD), mean squared deviations (MSD), variance component estimates, and the percentages of the total variance contributed by each component.

Source of Variation	df	SSD	MSD	Variance component	% Total variance
Among taxa	2	17.9	8.96	−0.34	−3.16
Among regions within taxa	20	239.7	12.0	1.5	13.97
Within regions	17	162.1	9.5	9.5	89.2

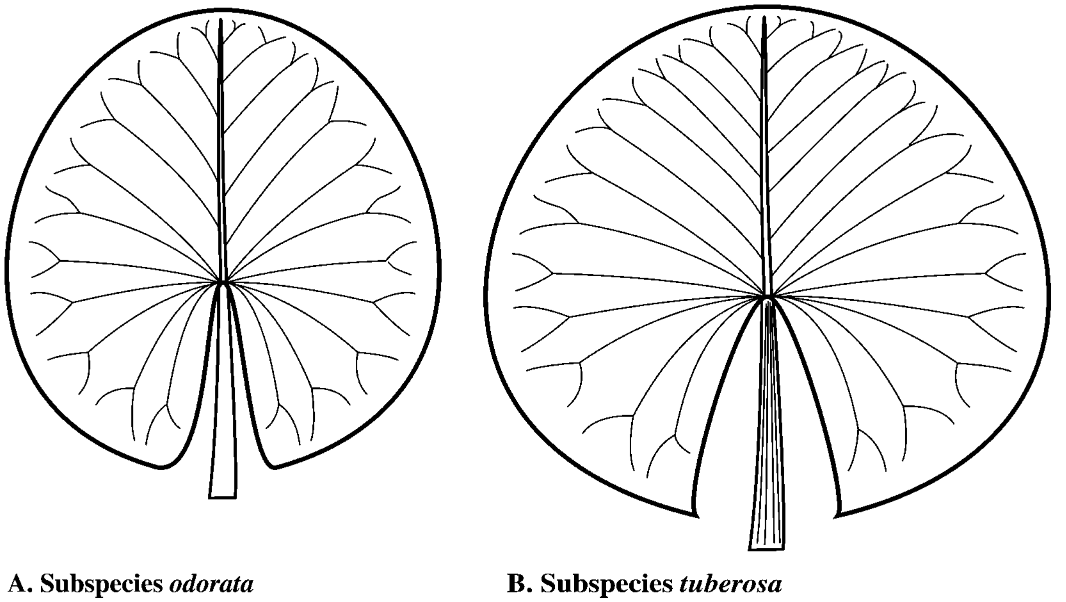

FIG. 5. Graphic illustration of the leaf blade from *N. odorata* subsp. *odorata* and *N. odorata* subsp. *tuberosa*. Illustration was generated based on data from four significant morphological characteristics: presence or absence of petiole striping, shape of lobe apex, mean length to width ratio and number of leaf veins.

sampled populations (length/width ratio 0.55–0.73, mean 0.63) , have a striped petiole, and a pointed lobe apex. In contrast, subsp. *odorata* has a more ovate leaf blade (length/width ratio 0.44–0.71, mean 0.56) , lack striped petioles, and have a rounded lobe apex.

The lack of grouping by subspecies or geography in the multivariate analyses (SAHN clustering, PCOA, and MST) may be due to overall high variability and possibly the use of a combination of flower and leaf blade morphology. In contrast, PCA results support a distinction between the subspecies and emphasize the importance of leaf blade characteristics (Fig. 2). Component one explains the majority of the variation found between the subspecies (36%) and is loaded high for quantitative leaf blade characteristics (Table 5). The distinction between the subspecies is further supported by discriminant analysis and PCA using only leaf blade characteristics. The discriminant analysis classified most samples to the correct subspecies, 71% for subsp. *odorata* and 67% for subsp. *tuberosa*. Therefore, it seems that characters other than those of the leaf blade may cause the blurring of boundaries between the subspecies.

Interpopulation Variation Using ISSR Markers. The ISSR data (UPGMA, PCOA, and MST) are congruent with the morphological analysis in showing clear distinctness between *N. mexicana* and *N. odorata* and in confirming the placement of those samples tentatively identified as *N. cf. mexicana*. Sample 42N collected from Charlotte County, Florida, appears isolated in PCOA analysis but linked to subsp. *odorata* with MST; the UPGMA analysis shows it grouping to *N. mexicana* but at very low coefficient, so that it may be considered as an individual cluster. The ITS gene tree (Woods et al. 2005) places sample 42N within subsp. *odorata* but bootstrap support is very low. The plant possesses some intermediate vegetative characters between *N. mexicana* and *N. odorata*, differing from *N. odorata* in having ascending, sparsely branched rhizomes and comparatively small leaves and flowers. Despite the isolated position of sample 42N unraveled with ISSRs, the ITS tree places it within subsp. *odorata*. Furthermore, polymorphic sites in ITS do not provide evidence for introgression with *N. mexicana* as in other samples of subsp. *tuberosa* (Woods et al. 2005). The UPGMA result could therefore be a spurious clustering with samples of *N. mexicana* at high distances.

Analyses of the ISSR markers with PCOA and MST (Fig. 3) show subsp. *odorata* to be a distinct entity with most of its samples interlinked in the MST. Although samples of subsp. *tuberosa* were separated on the first three PCOA axes, some MST linkage between samples of the two subspecies is evident. This may be due to gene flow between populations of the two subspecies. This hypothesis finds support from the ITS data (Woods et al. 2005). The UPGMA analysis (Fig. 4) did not separate the two subspecies into two clusters; nevertheless most of the clusters represent associations among samples of the same subspecies and sometimes reflect geographic relationships. Associations between some samples of subsp. *tuberosa* and subsp. *odorata* were also evident. These results reveal the underlying differential effectiveness of various algorithms at the population level, particularly the average linkage of UPGMA compared with the nearest neighbor linkage in the MST approaches. UPGMA groups taxonomic units based on average values (similarities or distances), whereas MST links the units based on their individual values.

In the UPGMA analysis, samples of subsp. *tuberosa* form three main groups, one of which encompasses three morphologically distinct samples from a single population in Michigan (Fig. 4). These samples could not initially be assigned to either subspecies based on morphological characters, but the ITS and *trnL-trnF* data suggest subsp. *tuberosa* (Woods et al. 2005). The second group includes three individuals from Ohio and Vermont near the eastern limit of the range of subsp. *tuberosa*. In these two groups, gene flow between the two subspecies is possible due to sympatry or parapatry. The third group of subsp. *tuberosa* comprises five samples from areas surrounding the Great Lakes, spanning the distribution of this subspecies. A sixth sample from Tennessee clustered last with this group; this population is found outside the main range of both subspecies and also occupies an isolated position in the ITS strict consensus tree (Woods et al. 2005). The remaining sample of subsp. *tuberosa*, that did not group with the others, represents an unusual pink-flowered form from southeastern Ohio, whose subsp. *tuberosa* identity is questionable.

Within subsp. *odorata*, three geographic groups are evident (Fig. 4). One group comprises samples from Texas, Louisiana, Georgia, and Michigan. The second includes samples from Vermont, Virginia, and Tennessee, and the third from Florida and Georgia. Several samples, all from eastern states, lie in clusters outside these three groups.

ISSR markers displayed high interpopulation variation in *N. odorata* (Table 3). Polymorphic bands range from 70–80% in subsp. *tuberosa*, and 82–100% in subsp. *odorata*. While subsp. *odorata* may appear more variable, the higher percentage of polymorphic bands may be caused by the larger sample size used for subsp. *odorata* (24 subsp. *odorata* and 13 subsp. *tuberosa*). Previous studies have shown that small sample sizes may underestimate the genetic diversity present within a species (Wolfe and Randle 2001). It is striking that the AMOVA reveals that most of the genetic variation in *N. odorata* is present within regions rather than between regions (Table 6). The geographically skewed partitioning, instead of the expected subspecies partitioning, might imply a highly variable species with boundaries that are relatively unrestricted to gene flow between regions. Such a pattern could be enhanced by gene flow between regions, perhaps via long-distance dispersal of propagules. Water-lily seeds are known to be ingested by waterfowl (Ridley 1930; Woodyard and Bolen 1984), creating the potential for such dispersal. Similarly, in red algal populations Hall and Vis (2002) attributed almost equal partitioning of variability within and between streams using ISSRs to possible long distance dispersal.

The high genetic variability of *N. odorata* is comparable to that found in other outcrossing organisms based on other markers (Huff et al. 1993; Hamrick and Godt 1996; Sales et al. 2001). Explanations therefore, could also lie in the breeding system. However, not much is known about the breeding system of *N. odorata*, including clonality or effective population size. Comparative analyses using the same ISSR primers in other *Nymphaea* species that reproduce largely by selfing might shed some light on the influence of reproductive systems on levels of genetic diversity. The high interpopulation variability in *N. odorata* could also be attributed to secondary contacts during repeated south-north migrations of populations caused by glacial advances/retreats that parallels the secondary contact hypothesis proposed by Stebbins (1985).

Based on morphological and ISSR information, this study does not support the recognition of *N. tuberosa* at the species rank. However, some support, particularly from PCOA and MST analyses of ISSR data, point to its recognition at subspecies level within *N. odorata*, as advocated by Wiersema and Hellquist (1994). Univariate and multivariate analyses of morphological data also show some distinction between the two subspecies. Based on these distinctions, a new set of morphological characters are proposed which have only the petiolar stripes in common with those proposed by Wiersema and Hellquist (1997).

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